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(19)

(54) STABLE SUPPORT MATERIAL FOR ENZYME INSOLUBILISATION

(71) We, KOCH-LIGHT LABORATORIES LIMITED, a British Company of 2 Willow Road, Colnbrook, Buckinghamshire, England, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to support materials to which biologically active macro-molecules, for example, proteins, enzymes and enzyme derivatives, can be attached to insolubilise them.

Our copending Patent Application No. 32911/72 (Serial No. 1,484,565) describes support materials to which biologically active molecules are linked by azo groups. These azo groups are stable but the support material from which the azo groups are derived are unsuitable and cannot be stored for long periods of time until the biologically active molecules have been attached. Accordingly these biologically active macro-molecules must therefore be attached to the diazo groups which become converted to azo linking groups as soon as the diazo groups are formed. Support materials having stablished diazo groups to which biologically active molecules can later be attached would clearly be advantageous and the invention has been made with this point in mind.

According to the invention there is provided a support material which is capable of bonding to biologically active macro-molecules to insolubilise them, which comprises a water insoluble solid onto at lest a part of whose surface has been absorbed a diazotised aromatic diamine, the diazo groups of the diazotised aromatic diamine having been converted to diazonium fluoroborate or fluoro-silicate groupings.

The support material of the invention can be used for insolubilising a wide variety of biologically active macro-molecules and has the advantage that when such molecules, e.g. enzymes, are insolubilised, the active sites of the enzymes are not often destroyed so that biological activity is retained.

The diazonium fluoroborate or fluorosilicate groupings of the support material of the invention are stable and the material can thus be stored in the dry state for considerable lengths of time before biologically active molecules are attached to it. This is clearly advantageous because it allows a manufacturer to produce a support material to which particular active molecules can be later attached for a particular application.

The support material of the invention can also be used for removing enzymes from, for example, effluents and fermentation media without prior activation.

When biologically active molecules have been attached to the support material the support material is biologically active, as are those described in our copending Patent Application No. 32911/72. (Serial No. 1,484,565).

The biologically active material produced can be used for catalysing reactions, and the active material easily separated from reaction products and reused as described in the above mentioned application.

The attachment of the biologically active molecules to the support material is sometimes almost quantitative and thus a solution containing a high concentration of biologically active molecules can be used to provide a carrier having a high concentration of molecules operating with high efficiency.

The biological activity of the biologically active material can thus be high.

Methods of reactivating the biologically active carrier material by a recoating procedure are described in copending Patent Application No. 32911/72 to which reference is made for a full description.

The stabilised diazonium salt groupings are fluorosilicate groupings or more preferably fluoroborate groupings. These groupings may be derived from, for example, fluorosilicic acid and fluoroboric acid respectively, or salts thereof, for example, sodium fluoroborate or fluorosilicate. The conversion of the unstable diazo groups to these stable diazonium salts can be effected at temperatures of about 0°C, for example 0 to 4°C, but has been successfully effected at 20°C with beneficial results with certain supports, for example, iron oxide and magnetic iron oxide.

Biologically active molecules, for example enzymes, can be coupled to the support material of the invention simply by mixing a solution of the enzyme with the material at a suitable pH at a low temperature, e.g. about 0°C, but can be effected at ambient temperatures. In certain cases with more stable enzymes, temperatures up to 80°C can be used since the diazonium salts are stable up to and beyond this temperature. Any diazo groups remaining after the attachment of the enzymes or other biologically active material can be made chemically inert by reaction with a phenol, for example β-naphthol as described in our copending Patent Application No. 32911/72 (Serial No. 1,484,565).

The water-insoluble solid can be, for example, in bead form, and can also be a prefabricated member such as a porous molecular sieve, tube, sheet, filter or membrane. When in bead form the material can be packed in columns.

As described in copending Patent Application No. 32911/72 the water-insoluble solid is generally any material which can be dyed or redyed with diazotised aromatic diamines, and often contains hydroxyl or polyamide groupings. Examples of particular solids are silica, e.g. sand, polysaccharide, cross-linked dextran (e.g. Sephadex), polyacrylamides, polyamides. polycarbonates, polyesters, glass (particularly porous glass) nylon, diatomaceous earth, natural or regenerated cellulose, for example paper, viscose rayon, Sigmacell 38, Whatman CC31, Whatman CF11, Neosyl, Celite, carboxymethyl cellulose, diethylamino-ethyl cellulose, Bioglas-1000, Biogel P-6, Enzacryl gel K_2, cellulose acetate, polyurethanes, Sephadex G—200, as well as inorganic hydrous oxides for example titanium oxide, zirconium oxide, aluminium oxide, iron oxide and magnetic iron oxide. Generally, all polar surfaces are dyeable, as are the surfaces of natural products such as cells of wood, e.g. balsa wood.

Biologically active molecules which can be attached to the support material of the invention include proteins, peptides, and entities containing these for example glycoproteins, mucoproteins, enzymes, hormones, antibiotics, antibodies, lectins and coenzymes, the tyrosine, histidine and other amino acids in, for example, peptides and proteins being capable of reacting with the diazo groups. Suitable enzymes are β-glucosidase, dextranase, amylase, glucamylase, catalase, glucose oxidase, thermolysin, N-acetyl amino acid amidohydrolase, peroxidase, chymotrypsin, uricase, pepsin, urease, pronase, lactate dehydrogenase, cholinesterase, glucose isomerase, isoamylase and pullulanase, which may be used alone or in any combination.

The diazotised aromatic diamine may be produced by diazotising an aromatic diamine for example diamino-benzene, preferably meta-diamino benzene. Preferably this compound is used in form of meta-diamino benzene HCl. The diazotisation reaction can be performed by reacting an aromatic diamine with an inorganic acid and sodium nitrite at a low temperature. The solution can then be warmed to 20°C and the precipitated solid used as a support itself after washing the fluoroboric acid.

One way of adsorbing the diazotised aromatic diamine on the surface of the water-insoluble support is to diazotise the aromatic diamine in the presence of the solid support. The diazotised aromatic diamine is strongly adsorbed on the surface of the support and can be freed from excess reactants by washing. This is the preferred process and after diazotisation the suspension can with certain supports be warmed to 20°C.

A second way of adsorbing the diazotised aromatic diamine on the surface of the support is to diazotise an aromatic diamine before adsorbing the diazotised aromatic diamine on the surface of the solid substrate.

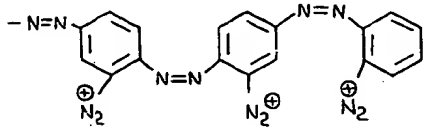
A third way is to absorb the aromatic diamine on the surface of the solid substrate, remove any excess diamine by washing, and then diazotise the aromatic diamine. Copending Patent Application No. 32911/72 (Serial No. 1,484,565)

describes in more detail the adsorption of diazotised aromatic diamine on a water-insoluble solid support. The resulting diazo groups can then be converted to stable diazonium salt groupings.

The support material of the invention with its stable diazonium salt groupings can be stored until use. It can be mixed with other materials to form powders, fibres, sheets, filters or beads for packing in columns.

The surface of certain materials, for example polypropylene and polyethylene cannot have diazotised aromatic diamines adsorbed on them. The support material of the invention can, however be incorporated in such polymers in their molten state, and the resulting mixtures formed into, for example, meshes, sheets, fibres and tubes, the surfaces of which can, if necessary, be roughened to expose the diazonium salt groupings. Alternatively the support material of the invention can be incorporated in certain paints, emulsion paints and surface coatings providing these do not contain groups which react with the diazonium groupings of the support and these are therefore subsequently free to react with the biologically active molecule to be immobilised.

When *m*-diamino benzene in acid solution is reacted with $NaNO_2$ at low temperatures, e.g. 0°C, a red brown solid, well known commercially as Bismark Brown is precipitated particularly if the solution is warmed to 20°C for 30 minutes. The polymer presumably has a structure similar to:



assuming all the amine groups are diazotised and can be stabilized by treatment with fluoroboric acid so that the ion of the acid is a counter ion and the product is Bismark Brown fluoroborate. Because of the presence of its diazonium groups diazotized Bismark Brown fluoroborate may be used in the preparation of support material of the invention for enzyme insolubilisation. β-D-glucosidase is very effectively insolubilized by attachment to this material; the activity detected on the solid represented 59% of the original activity of the soluble enzyme. While Bismark Brown fluorosilicate is also stable and useful, in marked contrast Bismark Brown in the form of its hydrochloride as the counter ion to the diazonium groups would be unstable.

The advantages of diazotised Bismark Brown in the form of its fluoroborate of fluorosilicate salt are these salts which can be absorbed onto dyeable materials of many diverse descriptions in a stabilized form which can be dried and thereafter reacted if desired at higher temperatures than heretofore with an enzyme to immobilise it.

The invention will now be illustrated with reference to the following Examples.

Example 1.

A mixture containing:

Cellulose CC31	0.100 g,

(chromatograph and column cellulose material, type 31, available from Whatmans Ltd.)

m-phenylene diamine HCl	0.050 g,
normal HCl	2.000 ml,

was stirred for 5 minutes at 4°C. *m*-Phenylene diamine HCl (Diamino benzene HCl) was used instead of *m*-phenylene diamine because it is more stable.

A 2% aqueous solution sodium nitrite (5.40 ml) was then added and the whole stirred for 30 minutes. The mixture frothed slightly and turned dark reddish brown.

The mixture was centrifuged and the supernatant liquid discarded. Glass distilled water (10 ml) was added and the whole stirred for 5 minutes. The tube was then centrifuged and the supernatant liquid discarded. Another aliquot of water

was added and the washing repeated. Washing was continued until the supernatant liquid was clear. Four washes were required.

The solid remaining was a pale brown and to this solid was added a 37.9% w/w solution of fluoroboric acid in water (10.00 ml). This was then stirred for 30 minutes and centrifuged, and the supernatant liquid discarded. The resulting cellulose-Bismark Brown solid was washed twice with water (10 ml aliquots), and then twice with 0.2 m sodium acetate buffer pH 5.0 (10 ml aliquots) in order to prepare it for enzyme coupling.

The enzyme employed in this and the following Examples 2 to 5 was β-glucosidase.

The cellulose-Bismark Brown support (100 mg) and B-Glucosidase (10 mg/ml acetate buffer 0.2 M, pH 5.0, 0.5 ml) were stirred together for 2 hours after which a saturated solution of sodium acetate and β-Naphthol (2.0 ml) was added. The mixture was stirred for 4.5 hours to anneal any uncoupled activity sites.

The mixture was then centrifuged and the supernatant liquid discarded. The solid was then washed alternately with sodium acetate buffer pH 5.0, 0.2 M and the same buffer containing 1 M sucrose and 1 M sodium chloride. This cycle was repeated three times and the mixture was then washed a further two times with sodium acetate buffer pH 5.0, 0.2 M, and finally the solid was suspended in 5 ml of the acetate buffer in preparation for assay.

The assay was as follows:— The substrate was *o*-nitrophenyl-β-D-glucopyranoside (2.0 mg/ml in acetate buffer pH 5.0, 0.2 M). 0.5 ml of the cellulose-Bismark Brown support β-glucosidase suspension was diluted ten times in acetate buffer and 0.5 ml was incubated at 37°C for 45 minutes with 3.0 ml of substrate solution. After this 0.5 ml of the mixture was added to 2.5 ml of sodium carbonate solution (0.2 M) to stop the reaction. The optical density of the supernatant after centrifuging was read at 420 nm against a water blank.

A sample of the soluble enzyme used was also assayed in the same way by incubation of 1 ml of a 0.002 mg/ml mixture with 3 ml of substrate for 45 minutes, and then taking 0.5 ml of the incubation mixture and adding it to 2.5 ml of sodium carbonate solution (0.2 M) and reading the optical density at 420 nm.

From the results the percentage activity, assuming all the enzyme to be coupled, was obtained. The results were as follows:—

		Optical Density
Soluble Enzyme 0.002 mg/ml	1.	0.163
	2.	0.155
Insolubilised Enzyme		
1st Preparation	1.	1.30
	2.	1.30
2nd Preparation	3.	1.26
	4.	1.34

From a calibration curve of mg of enzyme against optical density it was found that an optical density of 1.30 corresponded to 0.020 mg of β-Glucosidase. Since the theoretical amount of enzyme used in the suspension assay was 0.05 mg, the activity was 40.0%.

Example 2.

Example 1 was repeated but this time the cellulose-Bismark Brown support was dried before the enzyme was coupled. A mixture of:

Cellulose CC31	0.100 g,
m-phenylene diamine HCl	0.050 g,
normal HCl	2.00 ml

was stirred for 5 minutes at 4°C. A 2% aqueous solution of sodium nitrite (5.40 ml) was then added and the whole mixture stirred for 30 minutes. The mixture again frothed and turned a dark reddish brown.

The reaction mixture was then centrifuged and the supernatant discarded. Glass distilled water (10 ml) was added and the mixture stirred for 5 minutes. The tube when then centrifuged and the supernatant discarded. Another aliquot of water was added and the washing repeated until the supernatant was clear. This required four washes. The solid remaining was pale brown.

To the solid was added a 37.9% w/w fluoroboric acid solution in water (10.00 ml). This was then stirred for 30 minutes and centrifuged and the supernatant liquid discarded. The solid was washed three times with water (10 ml aliquots).

Methanol (anhydrous) 10.00 ml was added and the mixture stirred for 2 minutes. It was then centrifuged and the supernatant discarded.

The solid was mixed with diethyl ether (10 ml) and stirred for 2 minutes. (The ether had been previously redistilled and passed through an alumina column). The mixture was centrifuged and the solid was dried by passing air across it. A pale brown powder remained. This was the cellulose-Bismark Brown fluoroborate. It was stored at 4°C overnight and then coupled with β-glucosidase by stirring together for 2 hours the fluoroborate (100 mg) and β-glucosidase (10 mg/ml acetate buffer pH 5.0, 0.2 M, 0.5 ml) and then a saturated solution of sodium acetate and β-Naphthol (2.00 ml) was added. This was stirred for another 4.5 hours. The mixture was then centrifuged and the supernatant liquid discarded.

The mixture was washed alternately with acetate buffer (0.2 M, pH 5.0) and the same buffer containing 1 M sucrose and 1 M sodium chloride. Washing was carried out three times followed by a further two washes with acetate buffer. Finally the solid was suspended in 5 ml of acetate buffer (0.2 M pH 5.0).

The assay was carried out as in Example 1 and the optical densities obtained were as follows:—

		Optical density
Soluble enzyme 0.022 mg/ml	1.	0.14
	2.	0.13
Insolubilized enzyme		
1st Preparation	1.	1.23
	2.	1.18
2nd Preparation	3.	1.26
	4.	1.20

An optical density of 1.22 corresponded to 0.0188 mg of β-glucosidase and the activity was therefore 37.6%.

Example 3.

In this Example a solution of sodium fluoroborate in water, (47.4 g/100 ml water) was used instead of a fluoroboric acid solution.

The method employed was the same as that in Example 2, except that the fluoroboric acid was replaced by the sodium fluoroborate solution (10.00 ml). The enzyme coupling and assay was the same as that in Example 2.

The results were as follows:—

		Optical Density
Soluble enzyme 0.002 mg/ml	1.	0.13
	2.	0.14
Insolubilised enzyme		
1st Preparation	1.	1.10
	2.	1.10
2nd Preparation	3.	1.02
	4.	1.21

An optical density of 1.10 indicated that the % activity was 34%.
Clearly sodium fluoroborate was as effective as the free acid.

Example 4.

In this Example fluorosilicic acid was employed. The method employed was otherwise the same as that of Example 2. The results were as follows:

		Optical Density
Soluble enzyme	1.	0.13
	2.	0.13
Insolubilized enzyme		
1st Preparation	1.	0.35
	2.	0.33
2nd Preparation	3.	0.40
	4.	0.30

From the calibration curve the optical density of 0.35 was equal to 0.00525 mg enzyme, and the activity was therefore 10.5%.

Example 5.

Example 4 was repeated using sodium fluorosilicate instead of fluorosilicic acid. Since sodium fluorosilicate has a solubility of less than 1% in water a saturated solution was used.

The method employed was the same as that of Example 2 except that a saturated aqueous solution of sodium fluorosilicate (10.00 ml) was used instead of fluoroboric acid. The results obtained were as follows:—

		Optical Density
Soluble enzyme	1.	0.12
	2.	0.15
Insolubilized enzyme		
1st Preparation	1.	0.40
	2.	0.32
2nd Preparation	3.	0.50
	4.	0.38

From the calibration curve the optical density of 0.40 was equal to 0.006 mg enzyme, and the activity was therefore 12.0%.

The fluorosilicates therefore show some enzymic activity but this is not as good as that shown by fluoroborates.

Example 6.

Coupling at pH 7.0

Phosphate buffer (0.1 M, pH 7.0 60 cm^3) pre-cooled at 0°C was added to Bismark Brown cellulose fluoroborate (3.0 g). The mixture was stirred then centrifuged and the supernatant discarded. Papain solution (15 cm^3) obtained by taking the supernatant of a centrifuged suspension of crude papain (10 mg/cm^3) in activating buffer (a solution of 5×10^{-3}M cysteine hydrochloride and 2×10^{-3}M ethylene diamine tetracetic acid (EDTA) in 0.1 M, pH 7.0 phosphate buffer) was added to the solid and the suspension magnetically stirred at 4°C. After two hours a saturated solution of β-naphthol in saturated sodium acetate solution (60.0 cm^3) was added and the suspension stirred at 4°C for 4.5 hours. After centrifuging and

discarding the supernatant liquid the solid was washed alternately five times with activating buffer (60 cm^3) and a sucrose-salt solution in the same buffer (1.0 M in sucrose and 1.0 M in sodium chloride; 60 cm^3). The solid was then washed twice with a solution adjusted to pH 6.5 with 1.0 M NaOH of cysteine hydrochloride (10^{-3} M) and EDTA (4×10^{-4} M) in water (60 cm^3).

Finally the solid was suspended in a solution of sorbitol (1% w/v) in the same buffer solution (30 cm^3) and freeze dried.

Coupling at pH 4.5

The same method of preparation was used as above except that papain was added in a solution of cysteine hydrochloride (5×10^{-3} M) and EDTA (2×10^{-3} M) in acetate buffer (0.1 M, pH 4.5).

Coupling at pH 9.0

The same method of preparation was used as above except that papain was added in a solution of cysteine hydrochloride (5×10^{-3} M) and EDTA (2×10^{-3} M) in borate buffer (0.1 M, pH 9.0).

Preparation of Immobilized Papains at different pH's of coupling with Zn^{2+} present

For each respective pH of coupling the procedure is exactly as described above, except that firstly EDTA was omitted from all solutions apart from those used for the last two washes and freeze drying, and secondly the papain solutions added also contained zinc sulphate (1.35×10^{-3} M).

Preparation of Inactive Immobilized Papains

For each respective pH of coupling the procedure is exactly as described in the previous paragraph except that EDTA was omitted from all solutions used in the preparation and in freeze drying.

Determination of the Retention of Activity against N-α-benzoyl arginine ethyl ester hydrochloride (BAEE) for Immobilized Papains

A potentiometric determination of the acid produced during the hydrolysis at 25°C and pH 5.5 of N-α-benzoyl arginine ethyl ester hydrochloride was used to assay the enzymes. For the assay of papain, activating diluent (a solution of 10^{-3} M cysteine hydrochloride and 4×10^{-4} M EDTA, adjusted to pH 5.5 with 0.1 M sodium hydroxide solution, 0.90 cm^3) was placed in a cuvette surrounded by a water jacket kept at 25°C. To this was added substrate (0.05 M N-α-benzoyl arginine ethyl ester hydrochloride in activating diluent, adjusted to pH 5.5, 1.0 cm^3) and the solution stirred magnetically. The number of millilitres of sodium hydroxide solution (0.1 N) required per minute to maintain the pH at pH 5.5 were automatically recorded to give the blank rate of addition. Finally papain solution (the supernatant of a centrifuged suspension of crude papain 14.7 mg/ml in activating diluent; (0.10 cm^3) was added and the rate of addition of alkali recorded. The difference between this and the blank rate of addition is used to calculate the enzyme activity according to the formula below:

$$\text{activity units/mg of enzyme} = \frac{\text{difference in rates } (cm^3/\text{min}) \times 0.1 \times 1000}{\text{mg of enzyme}}$$

The assay solution for the assay of immobilised papain contained activating diluent (1.0 cm^3) and substrate (1.0 cm^3). After obtaining a blank rate of addition of alkali, solid immobilised papain (50 mg) was carefully added and the suspension magnetically stirred, while the rate of alkali addition required to keep the pH at pH 5.5 was determined.

The calculated retentions of activity are shown below.

Retention of Activity against BAEE for Immobilised Papains

	% retention of Activity	
pH coupling	coupled in absence of Zn^{2+}	coupled in presence of Zn^{2+}
4.5	9.04	16.0
7.0	13.8	24.0
9.0	12.0	17.0

Papain activity = 0.286 unit/mg of crude enzyme.

Regeneration of Activity by Removal of Zn^{2+} from Inactive Immobilised Papains after Freeze Drying

Samples (50 mg) of each of the three freeze dried inactive immobilised papains, were assayed for activity against BAEE as described, except that EDTA was omitted from the incubation mixture.

Other samples (50 mg) of each of the three freeze dried papains were washed twice with a solution adjusted to pH 6.5 with 1.0 M NaOH of cysteine hydrochloride (10^{-3} M) and EDTA (4×10^{-4} M) in water (0.5 cm^3) and once in a solution of sorbitol (1% w/v) in the same buffer solution (0.25 cm^3). After centrifuging the suspension and discarding the supernatant, each sample was suspended in a solution (1.0 cm^3) of cysteine hydrochloride (10^{-3} M) and EDTA (4×10^{-4} M) in water and assayed for activity against BAEE at pH 5.5 and 25°C under the same conditions of substrate, cysteine hydrochloride and EDTA concentration as described. The effect of this wash procedure is shown below.

Regeneration of Activity by Removal of Zn^{2+} from Inactive Immobilised Papains after freeze drying

pH of coupling	% retention before EDTA wash	% retention after EDTA wash
4.5	3.92	14.1
7.0	7.11	16.2
9.0	6.38	12.7

Determination of the Retentions of Activity against Casein for different immobilised Papains

The method of assay against casein as substrate was as follows: casein (1.0 cm^3, 1% w/v) in a solution of cysteine hydrochloride (10^{-2} M) and EDTA (4×10^{-4} M) in phosphate buffer (0.1 M, pH 6.5) was added to enzyme solution (1.0 cm^3) in the same buffer and the solution obtained incubated at 37°C. After 10 minutes the reaction was terminated by adding aqueous trichloroacetic acid solution (3.0 cm^3, 5% w/v). The resultant suspension was left at room temperature for 20 minutes and then centrifuged. The acid soluble peptides released by digestion were determined by measuring the O.D. at 280 nm of the supernatant solution.

A blank reading was obtained by carrying out the same procedure without enzyme present.

A calibration curve of increase in O.D. at 280 nm against enzyme concentration was constructed.

The procedure for the assay of immobilised papains was the same except that substrate was added to a suspension of insoluble enzyme (50 mg/cm^3, 1.0 cm^3), and the resultant suspension was well agitated during incubation. Retentions of activity were calculated by comparison with the calibration curve O.D. 280 nm rise against enzyme concentration for papain.

Retention of Activity of Immobilised Papains against Casein as Substrate

pH coupling	% retention of activity	
	coupled in absence of Zn^{2+}	coupled in presence of Zn^{2+}
4.5	1.94	2.18
7.0	2.43	3.22
9.0	1.84	2.84

The pH activity profiles at low and high ionic strength of immobilised papains coupled at different pH's

This experiment was carried out on all three preparations prepared at different pH's with Zn^{2+} present.

For the determination of the pH activity profiles of the papains, the same assay method and conditions described above were used except that the activating diluent and substrate solution are adjusted to the required pH of assay with 0.1 N NaOH and the pH stat apparatus set to titrate to the same pH. Activity in the pH ranges pH 3.5 to 8.5 was measured. Also the ionic strengths of the assay media were controlled by the concentration of sodium chloride present. The assay media for assays at low ionic strength contained no sodium chloride whereas the assay media for assays at high ionic strength were 1.0 M in sodium chloride.

Each pH activity profile was carried out over a single day to minimise errors due to changing intrinsic activity.

pH activity profile at low ionic strength for papain coupled at pH 4.5 (I~0.05)

pH of assay	Units/mg $\times 10^3$	% retention
3.5	2.42	17.9
4.5	3.08	22.8
5.0	3.85	28.5
5.5	3.43	25.4
6.0	3.94	29.2
6.5	4.84	35.8
7.5	1.70	12.6

pH-activity profile at low ionic strength for papain coupled at pH 7.0 (I~0.05)

pH of assay	Units/mg $\times 10^3$	% retention
3.5	1.69	12.5
4.5	2.25	16.6
5.0	2.88	21.2
5.5	3.77	27.8
5.5	3.36	24.7
6.0	3.76	27.7
6.5	3.11	22.9
7.0	1.89	13.9

pH-activity profile at low ionic strength for papain coupled at pH 9.0 (I~0.05)

pH of assay	Units/mg $\times 10^3$	% retention
3.5	1.91	14.2
5.0	3.93	29.3
5.5	4.20	31.3
6.0	4.25	31.7
6.5	5.07	37.8
7.5	2.79	20.8

pH-activity profile at high ionic strength for papain coupled at pH 4.5 (I~1.05)

pH of assay	Units/mg $\times 10^3$	% retention
3.5	0.00	0.00
4.0	1.86	13.8
5.0	3.03	22.4
5.5	3.47	25.7
6.0	3.10	22.9
6.5	3.32	24.6
7.0	2.22	16.4
7.5	2.21	16.4
8.0	1.72	12.7
8.5	1.05	7.77

pH-activity profile at high ionic strength for papain coupled at pH 7.0 (I~1.05)

pH of assay	Units/mg $\times 10^3$	% retention
4.0	1.86	13.7
5.0	2.84	20.9
5.5	3.48	25.6
6.0	3.84	28.3
6.5	2.35	17.3
7.0	1.89	13.9
7.5	1.75	12.9
8.0	1.36	10.0
8.5	1.09	8.00

pH activity profile at high ionic strength for papain controlled at pH 9.0 (I~1.05)

pH of assay	Units/mg $\times 10^3$	% retention
3.5	1.72	12.8
4.0	2.65	19.7
5.0	3.22	24.0
5.5	3.12	23.3
6.0	3.67	27.4
6.5	3.53	26.3
7.0	1.36	10.1
7.5	1.77	13.2
8.0	1.27	9.44

Example 7.

Support: Hercules HR350 — Magnetic Iron Oxide Bismark Brown Fluoroborate.

The support was prepared following the method hereinafter described in Example 11 substituting the Alumina-G2 with 50 g of Hercules MR 350 magnetic iron oxide.

Samples (100 mg) of the iron-oxide Bismark fluoroborate required only two buffer washes in ice-cold acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M), prior to coupling with α-amylase (10 mg/ml — 500 μl) in the buffer used at the wash stage. Annealing of each aliquot with β-naphthol (saturated, in saturated sodium acetate — 2 ml) followed for a further 2 hours at 4°C. The solids were subjected to five cycles of alternate washes in volumes (5 ml) of (a) acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M) at 0°C and (b) the same buffer with added sucrose (1.0 M) and sodium chloride (1.0 M). Prior to assay, the preparations were washed a further three times, and then suspended in a total volume (5 ml) in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).

Assay for α-Amylase Activity

Substrate: 1% (w/v) soluble starch in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).

Reagent: 3,5-Dintrosalicyclic acid (0.25 g).
2N Sodium Hydroxide (50.0 ml).
Rochelle Salt (75.0 g).
Dissolved in a total volume of 250 ml with distilled water.
Stored away from light.

Enzyme Standard: α-Amylase (100μ g/ml) in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).

Assay: Aliquots (100μ l) of soluble α-amylase standard, and iron oxide Bismark Brown fluoroborate α-amylase, were taken for assay. The samples were treated with substrate solution (5.0 ml). At zero time and at six minutes intervals over a 24 minute period of incubation of 37°C, aliquots (100μ l) were taken into Reagent (2.5 ml). The collected samples were heated in a boiling water bath for 10 minutes; and reading made at 570 nm after cooling to room temperature.

Readings O.D. 570 nm

α-Amylase sample	0 min	6 min	12 min	18 min	24 min
Blank	0.096	0.097	0.099	0.097	0.096
Soluble standard	0.132	0.213	0.272	0.334	0.403
Iron oxide Bismark Brown fluoroborate	0.174	0.223	0.339	0.428	0.499

The percentage of the enzyme offered to each aliquot which had coupled and remained active, was determined by comparing the rates of reaction during 6 to 24 minutes incubating between the tests and standard α-amylase samples.

Standard α-amylase (100μ g/ml)

Increase between 6 and 24 minutes = 403—213

= 0.190

Iron oxide Bismark Brown Fluoroborate α-amylase-Theoretical maximum 1000μ g/ml

Increase between 6 and 24 minutes = 499—223
= 276

Thus, the concentration of α-amylase present in the suspension is

$$\frac{276}{190} \times 100\mu \text{ g/ml},$$

$$= 145.2\mu \text{ g/ml, and}$$

$$\text{The proportion coupled} = \frac{145.2}{1000} \times 100\%$$

$$= 14.52\%.$$

Example 8 (Comparative).

Enzyme: Thermostable α-Amylase, Solusize N400, Mayvil Chemicals Ltd.

Bismark Brown fluoroborate (100 mg) prepared as described above was suspended in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M). The suspension in buffer (5 ml), at 0°C, was centrifuged and the supernatant was removed. This wash procedure was repeated once. The drained solid was mixed with an aliquot (300μ l) of acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M), and an aliquot (200μ l) of α-amylase solution containing 4.8 mg of total protein. The suspended support material was then left for 2 hours at 4°C. Identical preparations were made and left to couple at 20°C, 37°C, 50°C and 60°C for the same length of time. After coupling, an aliquot (2.0 ml) of β-naphthol (saturated, in saturated sodium acetate solution) was introduced into each suspension, and these were left for a further 2 hours, all at 4°C. The solids were then subjected to five cycles of alternate buffer washes at 0°C, using (a) acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M), and (b) this buffer containing sucrose (1 M) and sodium chloride (1 M). Finally three more washes using buffer (a) were applied. Aliquots of 5 ml were used throughout the wash procedure. Prior to assay, each solid was suspended in a total volume of 10 mls in buffer (a).

Assay for α-Amylase Activity

Substrate: 1% (w/v) soluble starch in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).

Reagent: 3,5-Dinitrosalicyclic Acid (0.25 g).
2N Sodium Hydroxide (50.0 ml).
Rochelle Salt (75.0 g).
Dissolved in a total volume of 250 ml in distilled water, and stored away from light.

Enzyme α-Amylase (N400) — 48μ g/ml of total protein in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).

Method: Aliquots (100μ l) were taken from each stirred suspension and from the soluble enzyme standard. At zero time, an aliquot of substrate (5.0 ml) was added to each stirred sample at 37°C. Aliquots Aliquots (100μ l) were taken into Reagent (2.5 ml) at 7 minute intervals over a 21 minute period. The samples were heated for 10 minutes on a boiling water bath, cooled, and centrifuged. Readings were made at 570 nm.

Results: The percentages of protein coupled to the supports over the temperature range used were determined by comparing the activity of the standard soluble α-amylase and the insolubilised enzyme activity.

Temperature of Coupling Bismark Brown Fluoroborate/α-amylase (°C)	percentage of coupling
4	12.7
20	13.8
37	14.0
50	13.5
60	8.26

Example 9 (Comparative).
Bismark Brown fluoroborate

Meta diamino benzene dihydrochloride (203 g, 1.15 moles, 2.3 equiv) was dissolved in water (4.8 litres), hydrochloric acid (480 ml, ~5 moles) added and the solution cooled to 0°C. Sodium nitrite (296 g, 4.3 moles) dissolved in water (3 litres) was added over approximately 30 minutes, with stirring, maintaining the temperature at 0(±2)°C, with external ice/salt or glycol/ cardice (solid carbon dioxide) cooling.

The mixture was then warmed to 20°C over approximately 30 minutes, small amounts of acetone being added in portions, at intervals, to minimise frothing. The mixture was stirred at about 20°C for ¾ hour, filtered and the solid washed on the filter several times with water, until the washings were almost colourless.

The solid was transferred to fluoroboric acid (1.8 litres) and stirred occasionally over about 1 hour. The solid was then filtered off, washed with water until the washings were colourless, washed with ether several times and air dried (yield 105 g, 42%).

Of six runs carried out, two on the above scale, three on a quarter scale, and one of 5 times the above scale, yields on the fluoroborate were fairly consistent at around 40%.

Stability

(i) The Bismark Brown fluoroborate from one run contained approximately 30% water; it was stored in this state for 8 weeks, then completely dried and its activity rechecked.
Original activity — 65.1% of β-glucosidase immobilised.
Final activity — 59.0% of β-glucosidase immobilised.

(ii) A second batch of fluoroborate was stored in a completely dry state for 6 weeks.
Original activity — 60.2% of β-glucosidase immobilised.
After 6 weeks — 55.4% of β-glucosidase immobilised.

Conclusions

The fluoroborate will insolubilise enzymes with the retention of a high activity; it appears stable on storage in a wet or dry state, and loses little activity on storage.

Example 10

Immobilisation of α-Amylase in Araldite and Emulsion Paint Films containing Bismark Brown Fluoroborate

Materials

α-Amylase — thermostable, solusize N400, Mayvil Chemicals Ltd.
Support — Bismark Brown Fluoroborate
Araldite — Hardner: HY 951 (1 vol), Ciba/Geigy.
Resin: MY 753 (9 vol).
Emulsion — Magicote, honey vinyl silk finish.

Method of Preparation of Films

Ground glass plates (1" × ½"), were coated with Araldite and with emulsion paint as follows. Araldite films were made by placing one small drop of hardner onto a glass slide, followed by nine larger drops of the resin. These materials were very thoroughly mixed on the plate using two glass rods, and the film was left to cure at 20°C for 16 hours. At this stage the film was still of a "tacky" consistency. Bismark Brown fluoroborate (5.0 mg) was evenly sprinkled onto the surface of the film using a small sieve. The film was left at 20°C for a further 24 hours until cured.

Emulsion paint films were made in a similar manner by spreading one large drop of paint to form an even film on a glass slide. The paint film was partially dried at room temperature for 30 minutes and Bismark Brown fluoroborate (5.0 mg) was applied to the surface as described (Type A). Films were also made (Type B) (not in accordance with the invention), applying two drops of paint and Bismark Brown fluoroborate (5.0 mg) to the slide and mixing the solid thoroughly with the paint. This formed a very thick dry film of uneven surface texture. This was also left at 20°C for a further 24 hours to ensure complete dryness. The plates were all washed at 4°C in distilled water for a minimum of 24 hours. This removed any solids not adhering to the film surface, and also removed excess chemical materials in the solid support.

Coupling of α-Amylase

An aliquot (100μ l) of α-amylase was diluted with acetate buffer (0.2 M, pH 5.8—400μ l) containing calcium chloride (0.01 M). This was applied to the film surface and left at room temperature for 2 hours. The solution was then tipped off the slide, which was placed in β-naphthol (saturated, in saturated aqueous sodium acetate — 3 ml). At intervals of 15 minutes the solutions were mixed on a vortex stirrer over a total period of 2 hours at room temperature. The films were then washed alternately in volumes (5 ml) of ice-cold buffers: (a) acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M) and (b) buffer a containing sucrose (1 M) and sodium chloride (1 M). Finally the slides were thoroughly rinsed in buffer a.

Assaying for α-Amylase Activity

Substrate — 1% (w/v) soluble starch in acetate buffer (0.2 M, pH 5.8) containing calcium chloride (0.01 M).
Reagent — 3,5-Dinitrosalicyclic Acid (0.25 g)
2N Sodium Hydroxide (50.0 ml)
Rochelle Salt (75.0 g)

Each film was placed in a test-tube with substrate (3.0 ml) at zero time and incubated at 37°C for 16 hours. Aliquots (100μ l) were taken from the substrate at zero for 16 hours. The samples were heated on a boiling water bath for 10 minutes and after cooling, readings were made at 570 nm.

Readings.

Sample	Zero	16 hours
Araldite	0.094	0.318
Emulsion A	0.094	0.217
Emulsion B	0.094	0.298

Example 11.

Meta-diamino benzene hydrochloride (42 g, 0.46 eqviv) was dissolved in water (1.1 litres), concentrated hydrochloric acid (100 ml) added and the solution cooled to 0°C. Alumina-G2 (alumina, grade 2 on the Brockmann activity scale available from Wohl Ltd.) (50 g) was added, followed by portionwise addition of sodium nitrite (60 g, 0.87 mole) in water (1 litre), with occasional hand-stirring and shaking, over approximately 20 minutes, at 0(±2)°C.

The mixture was maintained at 0°C for 30 minutes with occasional stirring/shaking then filtered. The solid was washed on the filter with ice-cold water until the washings were colourless, then transferred to fluoroboric acid (450 ml) at 0°C.

The mixture was hand stirred/shaken occasionally, at 0°C for about 30 minutes, then filtered.

The solid was washed with ice-cold water until the washings were colourless, then several times with ether and air dried. The product was in the form of pale grey/mauve granules (yield 53 g; activity 5.0%).

A run with titanium oxide was carried out diazotising at 0 to 5°C, warming to 20°C over 30 minutes and maintaining at 20°C, with occasional hand stirring/shaking for 30 minutes before filtration and subsequent work-up.

It was found that the product obtained by diazotising diamino benzene hydrochloride in the presence of nylon at 0°C decomposed on addition to fluoroboric acid. Consequently Bismark Brown fluoroborate-nylon was prepared by replacing the fluoroboric acid with an aqueous solution of sodium fluoroborate.

An experiment on the above scale was carried out using Celite 560, with continuous mechanical stirring.

Larger sale runs (2.5 kilos of support) have been carried out with alumina-G2, neutral alumina and titanium dioxide, using occasional hand stirring. The ratios of diamino benzene hydrochloride and other reactants to the support was reduced by one half, and the reactions effected at 0 to 5°C. The contact time between support and diazotised amine was greater than on the small scale; the nitrite addition took longer and filtration times were increased. It was found that the aluminas settled out from the reaction mixture reasonably well, and that most of the liquor containing some fines and fine black solid could be decanted from the bulk of the solid, which could be partially washed by decantation, with ice-cold water before a final filtration. However, with titanium dioxide, the product would not settle out completely, decantation was not possible, and a very slow filtration was carried out; consequently the titanium dioxide and diazotised amine were in contact, at 0 to 5°C, for about 6 hours.

Two large scale runs using Celite 560 (2.5 kilos) were attempted, with continuous stirring, using the ratio of reactants to support as on the small scale. The diazotised amine — Celite did not settle out fully and a fairly slow filtration was carried out. It was quit dark in colour (darker than the product prepared on a small scale) and further darkened on subsequent washing water water; in addition, continual water washing did not result in a colourless washing.

A third large scale run was carried out, with the ratios of diamino benzene hydrochloride and other reactants to the support reduced by half and using occasional hand stirring. The reaction was carried out below 0°C throughout, and faster, so that the Celite was in contact with the diazotised amine for 1 hour only. The Celite — diazotised amine product settled out quite well so that decantation was possible to a limited extent.

Results

Activity retained after coupling to β-glucosidase

Coated Support	Small scale		large scale	
	Colour	Average Activity (%)	Colour	Average Activity (%)
Alumina-G2	pale grey/mauve granules	5.0	Brown granules	10.8
Alumina-neutral	light brown	55.5	chocolate brown powder	33.2
Nylon	brown powder	42.1		
Celite	pale brown powder	14.5	pale brown powder	11.3
Celite (cont. stirring)	mauve-brown powder	29.5		
Titanium dioxide	pale mauve brown powder	10.5	mauve brown powder	19.2
Titanium dioxide (20°C)	dark mauve brown powder	30.7		

Stability

Bismark Brown fluoroborate — cellulose CC31 showed no loss in activity (~36%) after 8 months storage with respect to coupling to β-glucosidase.

Bismark Brown fluoroborate — titanium oxide showed no loss in activity (10.5%) after 7 months in respect to coupling to β-glucosidase.

A product kept for 11 weeks:

Original activity 10.06% of β-glucosidase immobilised,
After 11 weeks — 10.12% of β-glucosidase immobilised.

Bismark Brown fluoroborate — Celite 560 — a sample was retested after 8 weeks storage

Original activity — 14.5% of β-glucosidase immobilised,
After 8 weeks — 14.7% of β-glucosidase immobilised.

Bismark Brown fluoroborate — nylon — a sample was retested after keeping for 9 weeks.

Original activity — 42.1%,
After 9 weeks — 38.7%.

Method — Ordinary Iron Oxides.

Experiments were carried out both diazotising as above at 0°C then warming to 20°C on two iron oxides — Bayer types 130 and 180. The products were tested for enzyme activity using β-glucuronidase and pronase as the enzymes being coupled.

Results

Iron oxide type	Temp. (°C)	Average yield from 50g Fe_2O_3 (g)	Percentage average activity (Glucuronidase)	Percentage average activity (Pronase)
130	0	46	13.0	4.0
130	20	65	33.0	12.0
180	0	47	14.5	
180	20	66	46.0	

Magnetic Iron Oxides Methods

Small scale experiments (50 g of support) were carried out at 0°C with subsequent warming at 20°C for several hours, according to the methods described earlier, on three magnetic iron oxides — Fisons magnetic iron oxide and Hercules types HR350 and K300. In addition, an experiment was carried out, using HR350, diazotising at 1 to 12°C with subsequent warming to 18°C, and an experiment with K300, diazotising at room temperature, without cooling (temperature 12 to 18°C).

A large scale run (2 kilos of iron oxide) was effected with Fisons magnetic iron oxide at 20°C, following, closely, the method for Bismark Brown fluoroborate.

Results

Several of the Bismark Brown fluoroborate-magnetic iron oxides were tested in a damp state, and later fully dried and in some instances their activities re-checked all after coupling with β-glucosidase. A complete table of the results obtained are as follows:

Magnesium Iron Oxide (50g unless stated)	Temp. (°C)	Yield (g, dry weight)	Percentage activity tested (damp)	Percentage activity tested (dry)
Fisons	0	43	—	42.4
Fisons	20	46	42.7 (26% H_2O)	54.2
Fisons — 2 kilos	20	2570	—	22.9
HR 350	0	49.5	44.6 (10% H_2O)	—
K 300	0	48.5	36.1 (16% H_2O)	—
HR 350	20	68	51.8 (28% H_2O)	44.9
K 300	20	66	49.8 (29% H_2O)	—
HR 350	1–18	63.5	41.0 (27% H_2O)	20.5
K 300	12–18	62.5	36.2 (29% H_2O)	—

With ordinary iron oxides, the products prepared at 20°C gave higher enzyme activity than those prepared at 0°C. This was also true of the magnetic iron oxides tested, although, with these, differences were less significant.

It might be expected that at 20°C, a mixture of Bismark Brown fluoroborate-iron oxide and Bismark Brown fluoroborate would be formed; however, magnetic tests of Bismark Brown fluoroborate-magnetic iron oxide samples showed that the product, whether formed at 0°C or 20°C, was completely attracted to a magnet.

The words "Sephadex", "Sigmacell", "Whatman", "Neosyl", "Celite", "Enzacryl", "Hercules", "Araldite", "Magicote" and "Fisons" are registered Trade Marks.

WHAT WE CLAIM IS:—

1. A support material which is capable of bonding to biologically active macro-molecules to insolubilise them, which comprises a water-insoluble solid onto at least a part of whose surface has been absorbed a diazotised aromatic diamine, the diazo groups of the diazotised aromatic diamine having been converted to diazonium fluoroborate or fluorosilicate groupings.

2. A support material as claimed in Claim 1 in which the diazo groups of the diazotised aromatic diamine have been converted to diazonium fluorosilicate groupings.

3. A support material as claimed in Claim 1 in which the diazo groups of the diazotised aromatic diamine have been converted to diazonium fluoroborate groupings.

4. A support material as claimed in any of Claims 1 to 3 in which the water-insoluble solid is silica, a polysaccharide, a cross-linked dextran, a polyacrylamide, a polyamide, a polycarbonate, a polyester, glass, diatomaceous earth, natural or regenerated cellulose, nylon or an inorganic hydrous oxide.

5. A support material as claimed in any of Claims 1 to 3 in which the water-insoluble solid is wood.

6. A support material as claimed in any of Claims 1 to 5 in which the diazotised aromatic diamine is diazotised Bismark Brown.

7. A support material which is capable of bonding biologically active macro-molecules to insolubilise them substantially as herein described with reference to any of Examples 1 to 5.

8. A support material as claimed in claim 1 which is capable of bonding biologically active macro-molecules to insolubilise them substantially as herein described with reference to any of Examples 6, 7, 10 and 11.

9. A method of making a support material which is capable of bonding biologically active macro-molecules to insolubilise then in which an aromatic diamine is absorbed onto the surface of a water insoluble solid, before, during or after the absorption the diamine being diazotised, and the resulting absorbed-diazotised aromatic diamine is reacted with fluorosilicic acid, fluoroboric acid or a salt of fluorosilicic of fluoroboric acid.

10. A method as claimed in Claim 9 in which the reaction with fluorosilicic acid, fluoroboric acid or a salt of fluorosilicic of fluoroboric acid is effected at a temperature of 0 to 4°C.

11. A method of making a support material substantially as herein described in any of Examples 1 to 5.

12. A method of making a support material substantially as herein described in any of Examples 6, 7, 10 and 11.

13. A support material which has been made by a method as claimed in any of Claims 9 to 12.

14. A material as claimed in any of Claims 1 to 8 and 13 to which have been attached biologically active molecules to give a water-insoluble biologically active material.

15. A material as claimed in Claim 14 in which the biologically active macro-molecules are enzymes.

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